Neuroscience 284 (2015) 217–224

$GABA_A$ RECEPTOR-MEDIATED INPUT CHANGE ON OREXIN NEURONS FOLLOWING SLEEP DEPRIVATION IN MICE

T. MATSUKI,[a*†] M. TAKASU,[a] Y. HIROSE,[a] N. MURAKOSHI,[a,b] C. M. SINTON,[c] T. MOTOIKE[a] AND M. YANAGISAWA[a*]

[a] *International Institute for Integrative Sleep Medicine (WPI-IIIS), University of Tsukuba, Ibaraki 305-8575, Japan*

[b] *Cardiovascular Division, Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, Ibaraki 305-8575, Japan*

[c] *Arizona Respiratory Center, University of Arizona, AZ 85724-5030, United States*

Abstract—Orexins are bioactive peptides, which have been shown to play a pivotal role in vigilance state transitions: the loss of orexin-producing neurons (orexin neurons) leads to narcolepsy with cataplexy in the human. However, the effect of the need for sleep (i.e., sleep pressure) on orexin neurons remains largely unknown. Here, we found that immunostaining intensities of the α1 subunit of the $GABA_A$ receptor and neuroligin 2, which is involved in inhibitory synapse specialization, on orexin neurons of mouse brain were significantly increased by 6-h sleep deprivation. In contrast, we noted that immunostaining intensities of the α2, γ2, and β2/3 subunits of the $GABA_A$ receptor and Huntingtin-associated protein 1, which is involved in $GABA_A$R trafficking, were not changed by 6-h sleep deprivation. Using a slice patch recording, orexin neurons demonstrated increased sensitivity to a $GABA_A$ receptor agonist together with synaptic plasticity changes after sleep deprivation when compared with an *ad lib* sleep condition. In summary, the GABAergic input property of orexin neurons responds rapidly to sleep deprivation. This molecular response of orexin neurons may thus play a role in the changes that accompany the need for sleep following prolonged wakefulness, in particular the decreased probability of a transition to wakefulness once recovery sleep has begun.

Key words: orexin/hypocretin, sleep homeostasis, GABA, insomnia, receptor.

*Corresponding authors at: International Institute for Integrative Sleep Medicine (WPI-IIIS), University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan. Tel: +81-29-853-3301.
E-mail addresses: matsukit@gmail.com (T. Matsuki), yanagisawa.masa.fu@u.tsukuba.ac.jp (M. Yanagisawa).
† Present address: Development & Medical Affairs Division, GlaxoSmithKline K.K., Tokyo 151-8566, Japan.

Abbreviations: ACSF, artificial cerebrospinal fluid; BZDs, benzodiazepines; CNS, central nervous system; eGFP, enhanced green fluorescent protein; GABA, γ-aminobutyric acid; $GABA_AR$, $GABA_A$ receptor; $GABA_BR$, $GABA_B$ receptor; HAP1, Huntingtin-associated protein 1; LH, lateral hypothalamus; MCH, melanin-concentrating hormone; mIPSCs, miniature inhibitory post synaptic currents; NLGN2, neuroligin 2; NREM, non-rapid eye movement; PFA, paraformaldehyde; SD, sleep deprivation; SEM, standard error of the mean; TTX, tetrodotoxin.

http://dx.doi.org/10.1016/j.neuroscience.2014.09.063
0306-4522/

INTRODUCTION

Orexins (orexin A and B, also known as hypocretin-1 and hypocretin-2, respectively) are bioactive peptides, which are produced exclusively in the lateral hypothalamus (LH) in mammalian brains and which have been shown to play a critical role in regulating vigilance state transitions (see reviews: (Sakurai, 2007; Sinton, 2011)). The importance of orexin in the maintenance of consolidated bouts of sleep and wakefulness has been convincingly demonstrated by the fact that the sleep disorder narcolepsy with cataplexy is caused by a deficiency in orexin or orexin-producing neurons (orexin neurons) in humans and animals (Chemelli et al., 1999; Nishino et al., 2000; Thannickal et al., 2000). The activity of orexin neurons is increased during wakefulness and decreased during sleep (Lee et al., 2005; Takahashi et al., 2008). Furthermore, acute optogenetic activation of orexin neurons enhances the probability of a transition from sleep to wakefulness (Adamantidis et al., 2007), and chronic stimulation or inhibition of orexin neurons leads to a corresponding alteration in the vigilance state (Sasaki et al., 2011). Orexin neurons receive multiple afferents from several brain regions, including the limbic system, preoptic area, and monoaminergic neurons, and *in vitro* studies show that the activity of orexin neurons is regulated by several neuropeptides and neurotransmitters (Yoshida et al., 2006; Sakurai and Mieda, 2011). In summary, although the activity of orexin neurons is known to affect the continuity of sleep and wakefulness states, the influence of prolonged wakefulness on the properties of orexin neurons has not been characterized.

γ-Aminobutyric acid (GABA) is the primary and most abundant inhibitory neurotransmitter in the central nervous system (CNS). Sleep-active, GABAergic neurons in the preoptic area/anterior hypothalamus, in addition to local GABAergic interneurons, densely innervate the LH, including direct innervation of orexin neurons (Steininger et al., 2001; Uschakov et al., 2006; Hassani et al., 2010). Endogenous GABA release increases in the LH area during non-rapid eye movement (NREM) sleep (Nitz and Siegel, 1996; Alam et al., 2010). GABA affects sleep regulation via two pharmacologically distinct receptors, the $GABA_A$ receptor ($GABA_AR$) and $GABA_B$ receptor ($GABA_BR$) (Möhler, 2010). In a previous study, we used genetically modified mice to determine the role of the $GABA_BR$ on orexin neurons in sleep regulation (Matsuki et al., 2009). The specific deletion of $GABA_BRs$ on orexin neurons led to an instability of orexin neuronal activity and an increment in membrane

conductance, resulting in severe fragmentation of vigilance states. However, the role of a $GABA_AR$-mediated inhibitory input on orexin neurons is poorly characterized. $GABA_ARs$ are pentameric hetero-oligomers and constitute ligand-gated chloride channels (Olsen and Sieghart, 2008). $GABA_AR$ subunits are encoded by 19 different genes that have been grouped into eight subclasses based on sequence homology (α1–6, β1–3, γ1–3, δ, ε, θ, π, ρ1–3). The pentameric assembly of these subunits constitutes the functional $GABA_AR$ and is dependent on the regional distribution and cell-type-specific expression of each subunit in the CNS (Fritschy and Möhler, 1995; Pirker et al., 2000). Subunits of $GABA_ARs$ on basal forebrain cholinergic neurons (Modirrousta et al., 2007), and on neurons in the perifornical hypothalamus (Volgin and Kubin, 2007; Volgin et al., 2014) are up-regulated following sleep deprivation (SD), indicating that $GABA_ARs$ likely play a role in the homeostatic regulation of sleep. However, the molecular function and dynamics of $GABA_ARs$ on identified orexin neurons under these conditions are unknown.

In this study, we examined the molecular and functional alterations of $GABA_ARs$ on orexin neurons following SD, i.e., after an increase in homeostatic sleep pressure.

EXPERIMENTAL PROCEDURES

Mice

All experimental procedures involving animals were approved by the Animal Experiment and Use Committee of the University of Tsukuba and were performed strictly in accordance with ''*Guidelines for proper conduct of animal experiments*'', from the Science Council of Japan. Orexin-enhanced green fluorescent protein (eGFP) transgenic mice on a BDF1 background (Yamanaka et al., 2003) were maintained on a 12-h light/dark cycle (lights on from 9:00 A.M. [zeitgeber time: ZT0] to 9:00 P.M. [ZT12]). All experiments (*ad lib* (Ad lib) sleep and SD conditions) were begun at ZT0 and the mice were allowed *ad libitum* access to food and water throughout. Mice were housed individually and were independently and randomly assigned to each sleep condition. However, environmental conditions (including temperature, humidity, extraneous noise etc.) were identical for each mouse. Mice in the SD condition were deprived of sleep for 6 h (ZT0–ZT6) by gentle handling using the previously reported technique (Suzuki et al., 2013). In contrast, as reported by Suzuki et al. (2013) and confirmed by our own visual observations, the Ad lib sleep group were asleep for about 70% of the 6-h test period. After 6 h Ad lib sleep or SD, mouse brains were processed for immunohistochemical and electrophysiological experiments as described below. Mice used for immunohistochemistry were 8–16 wks of age (Ad lib sleep group: $n = 8$; SD group: $n = 10$), and mice used for electrophysiology were 3–6 wks of age (Ad lib sleep group: $n = 14$; SD group: $n = 17$).

Immunohistochemistry

For immunohistochemical experiments, mice from each condition were processed independently and randomly while ensuring that environmental conditions and time of sacrifice were the same for each mouse. In this study, eGFP fluorescence-positive (eGFP+) cells were treated as orexin-producing cells because there are no eGFP+ cells without expression of orexin mRNA (Yamanaka et al., 2003) and we found here that over 80% of orexin-containing cells were eGFP+ (i.e., about 20% of these cells were below the fluorescence detection limit). Brains of mice were fixed in 2% paraformaldehyde (PFA) overnight. After fixation, brains were sectioned (50 μm coronal sections) and stained with antibodies for $GABA_AR$ α1 (EMD Millipore, Billerica, MA, USA; rabbit polyclonal, 1:500), $GABA_AR$ α2 (Synaptic Systems, Goettingen, Germany; rabbit polyclonal, 1:500), $GABA_AR$ β2/β3 (EMD Millipore; mouse monoclonal, 1:200), $GABA_AR$ γ2 (Synaptic Systems; rabbit polyclonal, 1:500), neuroligin 2 (NLGN2) (Santa Cruz Biotechnology Inc., Dallas, TX, USA; goat polyclonal, 1:50) and Huntingtin-associated protein 1 (HAP1) (Santa Cruz Biotechnology Inc.; goat polyclonal, 1:200), and visualized by Alexa Fluor 594-conjugated secondary antibodies (1:2000) (Invitrogen, Thermo Fisher Scientific Inc., Carlsbad, CA, USA). After staining, images were acquired by laser-confocal microscopy equipped with a digital camera (LSM 700 with Axio Imager.Z2 and 63× oil objective; Carl Zeiss Meditec AG, Oberkochen, Germany). Luminance measurements were performed by analytical imaging software, ZEN 2010 ver. 6 (Carl Zeiss Meditec AG). Mean intensities (photon counts/unit area) of Alexa Fluor 594 fluorescence from the top to the bottom of cell bodies were analyzed randomly using the Histogram module in the ZEN software (Carl Zeiss Meditec AG), i.e., whole-cell fluorescence was measured and we did not attempt separate measurements of membrane fluorescence. To adjust intra- and inter-procedural variance for staining independently, the intensity of the fluorescence in GFP ''negative'' cells from the same slice was measured as a reference in each sample. These measurements were performed independently by two observers for the slices on each slide and these data were then summed before analysis.

Slice preparation and patch clamp recording

Orexin-eGFP transgenic mouse brains were processed for electrophysiology as described previously (Matsuki et al., 2009) with minor modifications. Whole-cell patch recordings were performed for 2 h from ZT7 to ZT9. Reagents used were (in mM): slice preparation buffer: 210 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 10 $MgCl_2$, 0.5 $CaCl_2$, 26 $NaHCO_3$, 11 glucose, pH 7.4; Bath buffer (artificial cerebrospinal fluid: ACSF): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11 glucose, pH 7.4 (bubbled continuously with 95% O_2/5% CO_2), 290–310 mOsm; K-Cl pipette solution: 145 KCl, 10 HEPES, 0.5 EGTA, 2 $MgCl_2$, 0.5 Na_2GTP, 5 MgATP, with or without 1 QX314 (Sigma–Aldrich Japan K.K., Tokyo, Japan) pH 7.4, 290–310 mOsm. The liquid junction potential of these solutions was estimated at 4.1 mV and was corrected in the data. For recording of the muscimol dose response, brain slices were superfused with bath ACSF and muscimol (Sigma–Aldrich Japan K.K.) was applied to the bath solution at five concentrations increasing from

80 nM to 50 μM in a stepwise manner. Each slice was used for each dose response. When the response was not observed at a lower concentration, the same slice was then used to test the next higher concentration after over 20-min washout period. For recording of miniature inhibitory post synaptic currents (mIPSCs), we used bath ACSF containing 50 μM DL-AP5 (Tocris Biosciences, Bristol, UK), 20 μM CNQX (Wako Pure Chemical Industries, Osaka, Japan), and 1 μM tetrodotoxin (TTX) (Sigma–Aldrich Japan K.K.). Data were acquired using Axopatch 200B-Digidata 1322A and pClamp ver.8. (Molecular Devices LLC, Sunnyvale, CA, USA) and analyzed using Clampfit (Molecular Devices LLC) and Mini Analysis ver. 6 software (Synaptosoft, Decatur, GA, USA). To identify orexin neurons, fluorescence and infrared-Dodt gradient contrast images were obtained with a fixed stage upright microscope, Axio Examiner.D1 fitted with the AxioCam MRm high resolution camera and using Axiovision ver. 4 software (Carl Zeiss Meditec AG).

Statistics

To evaluate immunofluorescence intensity and electrophysiological data, we analyzed data using Student's unpaired *t*-test for comparisons of immunohistochemical intensities and mIPSCs, or a two-way ANOVA (Ad lib sleep, SD) followed by Bonferroni post hoc comparisons for the muscimol dose–response analysis (GraphPad Prism 5.0; GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as mean ± standard error of the mean (SEM) and differences were considered significant at $P < 0.05$.

RESULTS

Changes in the expression of $GABA_A$Rs on orexin neurons after sleep deprivation

To study the alteration in GABAergic input via $GABA_A$Rs on orexin neurons following SD, we sleep deprived adult orexin-eGFP transgenic mice for 6 h. Control group mice were allowed Ad lib sleep during this period. Immunohistochemically, we studied the expression of several $GABA_A$R subunits on orexin neurons, as well as molecules involved in $GABA_A$R trafficking and inhibitory synapse specialization. Based on previous reports that $GABA_A$R α1, α2, β2, β3, and γ2 subunits are highly expressed in the LH (Fritschy and Möhler, 1995; Winsky-Sommerer, 2009), we examined the expression of these subunits on orexin neurons from brains of mice in the SD group in comparison with those from mice in the Ad lib sleep group. The immunoreactivity of these subunits was detected on the cell bodies and proximal dendrites in GFP-positive orexin neurons as well as GFP-negative non-orexin neurons. Although we found no differences in the expression of the α2, γ2, and β2/3 subunits on orexin neurons of both groups (Fig. 1), the intensity of $GABA_A$R α1 subunit immunoreactivity was visibly enhanced in orexin neurons from mice of the SD group in comparison with those from the Ad lib sleep group (Fig. 1A). Quantitative analyses of the luminance intensities of subunit immunoreactivity showed that the intensity of $GABA_A$R α1 immunoreactivity was in fact significantly higher on orexin neuron cell bodies from mice of the SD group (Fig. 1B). Furthermore, after values were normalized by the intensity of the same target proteins on GFP negative neurons in the same photo view, values remained significantly higher on orexin neurons from the SD group (Fig. 1C). We also examined the expression of two molecules in orexin neurons, HAP1 and NLGN2, which are involved in $GABA_A$R trafficking and inhibitory synapse specialization, respectively. HAP1 is one of the $GABA_A$R trafficking molecules and is abundantly expressed in orexin neurons in mouse brain (Lin et al., 2010). The deletion of this gene in orexin neurons leads to reductions in food intake, body weight and locomotor activity (Lin et al., 2010). However, we did not observe any changes in the expression of HAP1 in orexin neurons from the SD group in comparison with those from the Ad lib sleep group (Fig. 1B, C). In contrast, NLGN2 expression was significantly increased in orexin neurons after SD (Fig. 1B, C). Neuroligins are postsynaptic cell adhesion proteins known to interact with presynaptic neurexins and postsynaptic PDZ domain-containing scaffolding proteins (Craig and Kang, 2007). Among five neuroligins, NLGN2 is exclusively localized to inhibitory synapses and is believed to specify inhibitory synapses (Varoqueaux et al., 2004; Chubykin et al., 2007).

Functional changes in orexin neurons following sleep deprivation

We examined alterations in the electrophysiological characteristics of orexin neurons from mice of the SD group in comparison with those from the Ad lib sleep group. We did not observe any significant difference between the basal activities of orexin neurons from mice of the two groups (Ad lib sleep: $n = 6$, 17 neurons; SD: $n = 6$, 18 neurons). Resting membrane potentials were −56.6 ± 1.2 mV versus −56.6 ± 0.4 mV, resting discharge frequencies were 3.3 ± 0.5 Hz versus 3.2 ± 0.1 Hz, and membrane capacitances were 14.8 ± 1.8 pF versus 14.6 ± 1.2 pF in orexin neurons of the Ad lib sleep versus SD groups, respectively.

We next examined the response of orexin neurons to the bath-applied $GABA_A$R agonist, muscimol, under whole-cell voltage clamping conditions (Vm = −50 mV). Although we observed changes in current in neurons from both groups following muscimol application at concentrations higher than 0.4 μM, the current amplitude following the same dose of muscimol (10 μM) was significantly higher in the SD group than in the Ad lib sleep group (Fig. 2A). The EC_{50} values from the dose–response curve were 10.1 ± 3.3 μM in the Ad lib sleep group and 5.7 ± 0.9 μM in the SD group (Fig. 2B). These results suggested that the sensitivity of orexin neurons to a $GABA_A$R agonist was enhanced by SD.

Alteration of mIPSCs on orexin neurons following sleep deprivation

In order to examine the synaptic plasticity of orexin neurons, we recorded mIPSCs on orexin neurons under

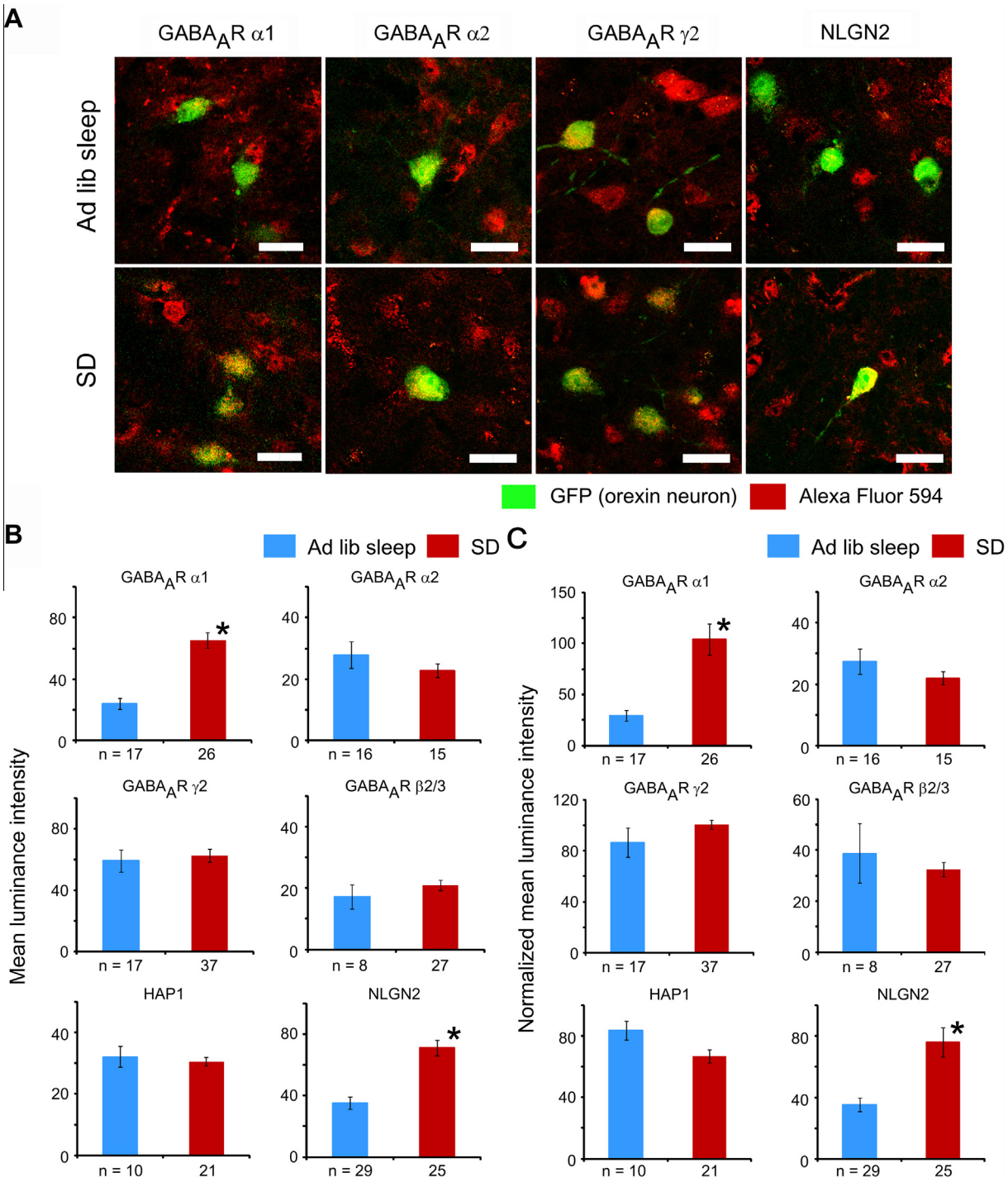

Fig. 1. Increased expression of $GABA_A$ receptor ($GABA_AR$) α1 subunit and neuroligin 2 (NLGN2) on orexin neurons after SD. (A) Representative images of the lateral hypothalamic area of brain slices labeled for orexin (green, GFP fluorescence derived from orexin-eGFP transgene) and $GABA_AR$ α1, $GABA_AR$ α2, $GABA_AR$ γ2, or NLGN2 immunoreactivity (red, Alexa Fluor 594) from mice of the Ad lib sleep and SD groups. Scale bars, 20 μm. (B) Mean luminance intensities (photon counts per unit area of target) on orexin neurons (eGFP+ cells), and (C) Normalized mean luminance intensities (relative values against luminance intensities of each target protein on GFP-negative cells) of $GABA_AR$ α1-, $GABA_AR$ α2-, $GABA_AR$ γ2-, $GABA_AR$ β2/3-, HAP1-, and NLGN2-immunoreactivity in orexin neurons from mice of the Ad lib sleep (blue bars) and SD (red bars) groups. The number of neurons which were examined in each condition is shown. Data are displayed as mean ± SEM. $^*P < 0.001$, by Student's *t*-test.

whole-cell voltage clamping (Vm = −50 mV). We could detect mIPSCs on orexin neurons by using blockers for NMDA receptors (DL-AP5), AMPA/kinate receptors (CNQX), and sodium channels (TTX). These mIPSCs were fully blocked by 30 μM bicuculline, suggesting that they were $GABA_AR$-mediated currents (data not shown).

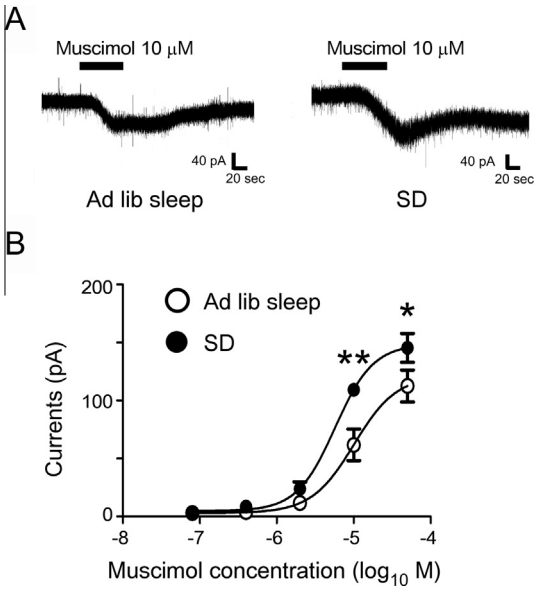

Fig. 2. Increased sensitivity of orexin neurons to a $GABA_AR$ agonist after SD. (A) Representative traces from whole-cell patch recording showing the effects of muscimol (10 μM, bath application) during voltage clamp (Vm = −50 mV) on the current change of an orexin neuron from mice of the Ad lib sleep and SD groups. Under these conditions (i.e., ACSF-bath and K-Cl pipette solutions), current changes were inward, as predicted. (B) Dose response curves of muscimol on whole-cell currents during voltage clamp (Vm = −50 mV) in orexin neurons from mice of the Ad lib sleep (open circles, $n = 8$, each dose) and SD (closed circles, $n = 8$, each dose) groups. Data are displayed as mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$ by a two-way ANOVA and Bonferroni post hoc test.

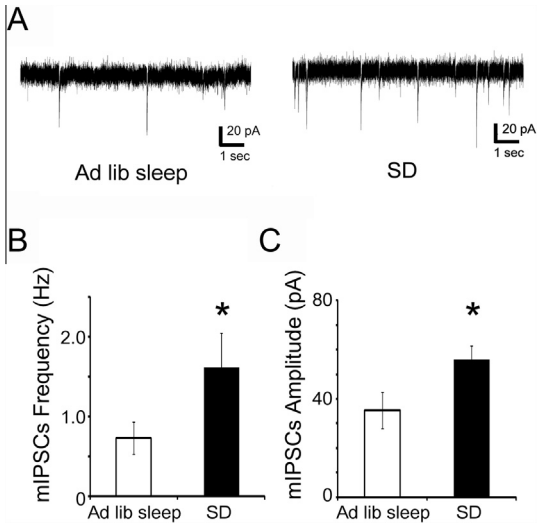

Fig. 3. Alteration of miniature inhibitory post synaptic currents (mIPSCs) in orexin neurons after SD. (A) Representative data for mIPSC recordings from whole-cell patch recording of orexin neurons from mice of the Ad lib sleep and SD groups during voltage clamp (Vm = −50 mV). Under these conditions (i.e., ACSF-bath and K-Cl pipette solutions), currents were inward, as predicted. mIPSCs were recorded in the presence of AP-5 (50 μM), CNQX (20 μM) and TTX (1 μM). Summary data for mean mIPSC (B) frequency and (C) amplitude from mice of the Ad lib sleep ($n = 7$) and SD ($n = 6$) groups. Data are displayed as mean ± SEM. $^{*}P < 0.05$ by Student's *t*-test.

We observed notable differences in frequency and amplitude of the mIPSCs on orexin neurons between the two groups (Fig. 3A). Both the frequency and amplitude of mIPSCs were significantly enhanced in orexin neurons from mice subjected to SD when compared to orexin neurons from Ad lib sleeping animals (Fig. 3B, C).

DISCUSSION

The orexin system is crucial for transitions between vigilance states and thus for maintaining uninterrupted periods of wakefulness and sleep (Sakurai, 2007). In this study, we have demonstrated, both immunohistochemically and functionally, that prolonged wakefulness affects the GABAergic modulation of orexin neurons through changes in $GABA_ARs$.

Expression of the α1 subunit of $GABA_ARs$ in orexin neurons after sleep deprivation

Many hypnotics, including benzodiazepines (BZDs), zopiclone and zolpidem, are known to target the α subunit of the $GABA_AR$. Endogenous agonist binding sites of $GABA_ARs$ are directly modulated by these drugs through allosteric mechanisms (Winsky-Sommerer, 2009; Rudolph and Knoflach, 2011). In this study, we observed the up-regulation of $GABA_AR$ α1 subunit expression in a specific functional nucleus following a period of extended wakefulness. $GABA_ARs$ containing the α1 subunit show higher affinities and efficacies for BZDs compared to other subunit-containing $GABA_ARs$ (Sieghart, 1995; Olsen and Sieghart, 2008). Thus the up-regulation that we have demonstrated here will positively modulate endogenous agonist effects and enhance GABAergic inhibitory currents in orexin neurons. Indeed, our electrophysiological data showed enhancement of the sensitivity for a $GABA_AR$ agonist in orexin neurons after SD. Immunohistochemical data did not distinguish those receptors located on the cell membrane or in the cytoplasm. However, it is likely that the enhancement of the sensitivity for a $GABA_AR$ agonist reflected an increase in function of these receptors on the cell membrane. These receptor alterations further suggest that orexin neurons express the plasticity necessary to adapt rapidly to ongoing changes in the need for sleep.

Our findings raise the possibility that SD might have increased the total number of functional $GABA_ARs$ on orexin neurons. However, we did not observe any differences between the Ad lib sleep and SD groups in the expression of the other four subunits of $GABA_ARs$ including the γ2 subunit. The γ2 subunit usually occurs in a one-to-one relationship with the α1 subunit and it is therefore surprising that we did not observe an increase in the γ2 subunit. However, another subunit in a non-α containing $GABA_AR$ could be replaced by the α1 subunit following SD. Furthermore, it is known that the majority (almost 90%) of $GABA_ARs$ contain the γ2 subunit (Rudolph and Knoflach, 2011), and hence any γ2 subunit changes could be relatively insignificant. Overall therefore, these results suggest that the total amount of $GABA_AR$ protein in each orexin neuron is unlikely to

be much affected by SD. Instead, it is reasonable to assume that subunit components of $GABA_AR$s were altered and thus the proportion of α1 subunit-containing $GABA_AR$s was increased following SD. One potential limitation of this conclusion is that our antibody for the β2 subunit did not differentiate the β3 subunit. This may be of particular importance because a previous report (Volgin et al., 2014), which found changes in $GABA_AR$ mRNA expression in the perifornical hypothalamus following SD in rats, reported that SD affected $GABA_AR$ β3 expression but not β2 expression in this region. This leaves open the possibility that we might have found $GABA_AR$ β3 subunit up-regulation after SD in the present study if our antibody had been sensitive to this differentiation. However, apart from possible species and regional differences, Volgin and colleagues did not examine $GABA_AR$ mRNA expression at the level of specific cell types (Volgin et al., 2014). Any potential changes to orexin neuron-specific α1 subunit expression in their study might therefore have been negated by potentially larger changes on a more numerous cell type in the region of study. And, for the same reason, we found no difference in $GABA_AR$ β2/β3 subunits because this change does not occur on orexin neurons following SD.

Modulation of synaptic plasticity by sleep deprivation

Postsynaptic current changes are known to reflect alterations in synapse number, presynaptic vesicle formation and release, or post-synaptic receptor modulation. While the change in the amplitude of mIPSCs indicates alterations in the postsynaptic component, including synaptic receptor sensitivity, the change in the frequency of mIPSCs is thought to result from modification to the presynaptic component of synaptic transmission or formation of inhibitory synapses (Turrigiano, 2012). Consequently, the change in the amplitude of the mIPSCs that we found here would be the basis for the enhancement of $GABA_AR$ agonist sensitivity in orexin neurons from the SD group. Recently, Ibata et al. reported that rapid changes in synaptic plasticity were induced by changes in postsynaptic firing in cortical neurons (Ibata et al., 2008). By extension, this suggests that the change of orexin neuronal activity during extended wakefulness might be sufficient to induce the postsynaptic changes we found here and therefore that the effect is not due to SD per se. In contrast, the change in the frequency of mIPSCs would be the consequence of increasing either the probability of presynaptic vesicle release or synaptic formation. Since GABA release is decreased in the LH during extended wakefulness (Nitz and Siegel, 1996; Alam et al., 2010), the enhanced frequency of mIPSCs found here indicates therefore that GABAergic synapse formation increased during SD.

Several studies have reported alteration in synaptic plasticity with changes in vigilance state (McDermott et al., 2003; Wang et al., 2011; Yan et al., 2011). However, most studies have focused on hippocampal and prefrontal cortical neurons and relatively little work has addressed changes in hypothalamic neurons, including orexin neurons. Orexin neurons remain active during SD (Modirrousta et al., 2005) and it is likely therefore that these neurons play a role, through innervation of all components of the arousal system, in maintaining sufficient alertness during long-term sleep loss. Rao et al. reported that excitatory postsynaptic currents were enhanced in mouse orexin neurons either by modafinil-induced prolonged wakefulness or by SD (Rao et al., 2007). These authors hypothesized that orexin neurons maintain their activity by means of excitatory synaptic plasticity changes to adapt to homeostatic sleep pressure (Rao et al., 2007). Similarly, the number of presynaptic output boutons of orexin neurons was increased during prolonged wakefulness in zebrafish (Appelbaum et al., 2010). Thus, enhanced presynaptic excitatory neurotransmitter release combined with changes to input mechanisms, including synapse scaling and receptor accumulation at postsynaptic sites, maintain orexin neuronal activity under these circumstances. Conversely, our findings now show that SD also concurrently alters the sensitivity of orexin neurons to inhibitory input by modulating $GABA_AR$s while GABA release from presynaptic sites is reduced during SD. This plasticity occurs over a duration that corresponds approximately to the time that sleepiness starts to intrude during prolonged wakefulness. This suggests that a postsynaptic inhibitory mechanism on orexin neurons is augmented during extended wakefulness, presumably in preparation for eventual sleep onset. If the orexin system plays an important role during extended wakefulness to maintain vigilance, we have shown here that it thus may also play a role *after* extended wakefulness. The latter mechanism reduces the probability of a transition to wakefulness once sleep onset has occurred and so favors the maintenance of sleep. Indeed, the latter has been confirmed in an optogenetic study (Carter et al., 2009), using a technique that manipulates the postsynaptic response. Interestingly, taken as a whole, these data suggest that it might eventually be possible to generate modulators for the orexin/GABA system at presynaptic or postsynaptic sites that have opposite functional effects, either excitatory or inhibitory, depending on the need for sleep.

The expression of NLGN2 was also markedly increased in orexin neurons after SD. A recent report demonstrated that NLGN2 interacts with gephyrin and collybistin, both of which are required for the differentiation of inhibitory synapses (Poulopoulos et al., 2009), and our present findings thus indicate that NLGN2 may modulate the inhibitory synapse on orexin neurons in response to SD.

Potential experimental issues

For immunohistochemical analyses, the absolute intensity of each marker was referenced to non-orexin cells (i.e., eGFP-negative neurons) to correct for intra- and inter-procedural variance. These referenced cells were selected randomly in the same slice and close to the orexin target cells. The mean intensity of each target protein and size of the non-orexin cells were found to be similar between Ad lib sleep and SD groups, with the exception of HAP1, which was significantly increased in the SD group (data not shown). However, the relative

intensity difference of HAP1 did not reach statistical significance (Fig. 1C). In the LH, there are multiple heterogeneous neuronal groups, containing, inter alia, GABA, glutamate, histamine, and melanin-concentrating hormone (MCH). Future studies, outside the scope of the present work, will characterize how synaptic modulation affects input to each of these types of neuron during SD.

Extended wakefulness is inevitably associated with increased motor activity and stress. Even a modest duration of SD results in a systemic stress response, including increased release of glucocorticoids and proinflammatory cytokines. We have previously reported that phosphorylated forms of dynamin 1 and N-myc downstream-regulated gene 2 were increased by 6 h of SD achieved by gentle handling even though these proteins were not influenced by a restraint stress (Suzuki et al., 2013). In that study, mice were subjected to the restraint stress for 30 min and glucocorticoids levels in the restraint stress group were higher than those of mice in the SD group. Here mice were exposed to an identical SD procedure and we can assume therefore that glucocorticoid levels were increased to a similar extent. We do not know, however, whether this level of stress may have directly affected $GABA_AR$s on orexin neurons. It is also unknown whether an increase in motor activity that is inevitably associated with SD could have altered the inhibitory input properties of orexin neurons. This is especially relevant for the orexin system since the discharge frequency of orexin neurons is increased in proportion to the intensity of active wakefulness (Lee et al., 2005; Takahashi et al., 2008). Further studies will therefore be required to confirm that the $GABA_AR$ change that we have identified results from SD and not a correlated but uncontrolled variable.

CONCLUSION

In this study, we have described a significant change to the input properties of orexin neurons during SD. This change has functional effects and indicates that orexin neurons respond to extended wakefulness with a relatively rapid change to $GABA_AR$ subunits that will favor the maintenance of more prolonged sleep bouts once sleep onset occurs. $GABA_AR$s on orexin neurons will therefore contribute to at least one of the mechanisms by which orexin modulates vigilance state transitions.

CONFLICT OF INTEREST

The authors declare that no competing interests exist.

AUTHOR CONTRIBUTIONS

T.Ma., M.T., Y.H., T.Mo., and M.Y. designed the research. T. Ma., M.T., and Y.H. performed the experiments and analyzed data. T.Ma., N.M., and M.Y. contributed to analytic tools. T.Ma., C.M.S., T.Mo., and M.Y. analyzed data and wrote the paper.

Acknowledgements—This study is supported by the Cabinet Office, Government of Japan through the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program) (M.Y.), the Ministry of Education, Culture, Sports, Science and Technology, World Premier International Research Center Initiative (M.Y.), and the Perot Family Foundation (M.Y.). M.Y. is a former Investigator of the Howard Hughes Medical Institute.

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(Accepted 27 September 2014)
(Available online 5 October 2014)